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Isolation of Human Tyrosinase from Cultured Melanoma Cells

A. WITTBJER,[1] B. DAHLBÄCK,[4] G. ODH,[1] A.-M. ROSENGREN,[2]
E. ROSENGREN[1] and H. RORSMAN[3]

Departments of [1]Pharmacology, [2]Biochemistry, and [3]Dermatology, University of Lund, and [4]Department of Clinical Chemistry, Malmö, Sweden

Tyrosinase was isolated from cultured melanoma cells using a procedure involving solubilization of the enzyme by means of Triton X-100, followed by different types of chromatography and tryptic digestion to make the enzyme soluble even in the absence of detergent. Starting with a membranous material containing 72 mg protein, 0.21 mg tyrosinase was obtained. The recovery of tyrosinase was 36% of the quantity found in the membranous starting material. In order to acquire a completely purified enzyme preparation suitable for amino acid sequence analysis, SDS–PAGE followed by blotting onto a polyvinylidene difluoride membrane was performed as a final step. The apparent molecular weight was found to be 66000. Determination of the amino acids of the aminoterminal portion by automated Edman degradation showed the following sequence: His-Phe-Pro-Arg-Ala- X -Val-Ser-Ser-Lys-Asn-Leu-Met-Glu-Lys-Glu- X - X -Pro-Pro- The enzyme purified has an amino acid sequence identical with that of human tyrosinase deduced from c-DNA by Kwon et al. (17). Striking similarities between our amino acid sequence and that predicted by Yamamoto et al. (18) from mouse tyrosinase c-DNA were also observed. ***Key words: Dopa; Cysteinyldopa; Melanin.***

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H. Rorsman, Department of Dermatology, Lasarettet, S-221 85 Lund, Sweden.

Tyrosinase is the enzyme responsible for melanin formation in the skin and some other tissues (EC. 1.14.18.1). The isolation of tyrosinase from mammalian tissues offers considerable difficulties. The yields are generally low. The enzyme only amounts to 0.1–0.3% of the total protein of the cultured melanoma cells, studied here. Even lower quantities of the enzyme, less than 0.01%, are reported to occur in pigmented melanomas (1). Tyrosinase exhibits great heterogeneity (2, 3) and is to a large extent anchored to the melanosome membrane (2, 4). It is therefore difficult to separate the enzyme from other membrane proteins. The heterogeneity observed on isoelectric focusing is probably due mainly to the different degrees of sialic acid substitution of the enzyme.

The different degrees of sialic acid substitution limit the use of methods which are based on the charge of the protein (3, 5). Moreover, no efficient method involving affinity chromatography has been developed. All in all, we have had substantial problems when trying to isolate tyrosinase in fairly good yields from cultured melanoma cells.

The object of the present work was to develop a convenient method for the preparation of tyrosinase from cultured melanoma cells. The aim was to get the enzyme in a form suitable for use in immunological studies and for characterizing its structure. A procedure for the isolation of tyrosinase based mainly on hydrophobic interaction chromatography of the enzyme has now been developed.

MATERIAL AND METHODS

Cultures of a pigment-producing human melanoma cell line (IGR-I) were obtained from Dr Christian Aubert, Marseilles, and have been kept in culture at the Tornblad Institute, University of Lund since March 1982 (6).

Protein determination: Protein was assayed *ad modum* Lowry et al. (7) in steps 1–4. Bovine serum albumin was used as a standard. In step 5 onwards the protein concentration was measured by Bio-Rad Protein Assay (Richmond, Calif., USA) since the fractions collected contained only small amounts of protein.

Tyrosinase determination: The measurement of tyrosinase activity was based upon the determination of stereospecific dopa oxidation by measurement of the quantity of 5-S-L-cysteinyl-L-dopa formed in the presence of D,L-dopa and L-cysteine. 0.1 ml of the eluate under investigation was added to 0.9 ml solution containing 1 mM L-dopa and 1 mM D-dopa, 3mM L-cysteine, 10 µg catalase (bovine liver, Sigma) in 0.5 M KH_2PO_4, pH 7.4. The presence of catalase in the incubate limits non-specific oxidation. Incubation was performed at 37°C for 2 min under gentle air bubbling. The reaction was stopped by adding 100 µl of the incubate to 900 µl of 0.4 M PCA. The content of 5-S-L-cysteinyl-L-dopa was determined by means of HPLC and electrochemical detec-

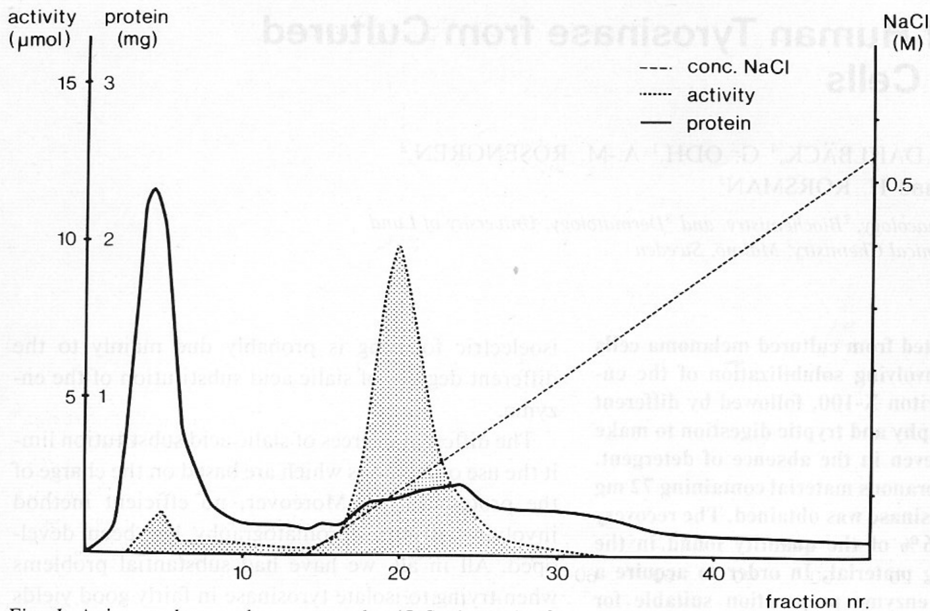

Fig. 1. Anion exchange chromatography (Q-Sepharose) of Triton X-100 solubilized tyrosinase from human melanoma cells. Activity and protein given per fraction.

tion (8). The activity was given as the sum of the 2- and 5-s-isomers, as dopa oxidized in the presence of cysteine gives both 5-S-cysteinyldopa and 2-S-cysteinyldopa. The formation of 5-S-cysteinyldopa predominates and is four times higher than that of 2-S-cysteinyldopa.

SDS–Polyacrylamide Gel Electrophoresis: An 10% SDS-PAGE slabgel (140 mm×129 mm×1.5 mm) was prepared *ad modum* Laemmli (9), using a Bio-Rad electrophoresis apparatus. Sample preparation: 50 µl of the sample was mixed with 50 µl of solubilization solution containing: 20% (w/v) glycerol, 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.125 M TRIS-HCl, pH 6.8, and small amounts of bromphenol blue, and boiled for 2 min.

As MW-markers for the Coomassie Brilliant Blue R-250 staining we used Pharmacia Electrophoresis Calibration Kit for low molecular weight proteins (Pharmacia, Sweden).

Running buffer (upper and lower): 0.083 M TRIS (1/3 of concentration used by Laemmli) 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3. Running conditions: 7 mA for 30 min, 25 mA for 5 h and 30 min.

Protein blotting: The blotting was performed with a semi-dry electroblotter apparatus (JKA-Biotech, Denmark). A PVDF (polyvinylidene difluoride)-membrane (Millipore, Bedford, Mass. USA) was prewetted in 100% methanol for 2 s, H_2O of Millipore quality for 2 min and 10 mM CAPS buffer, 20% methanol for 2 min, and laid on 9 sheets of filterpaper. The first 6 sheets were soaked in anodic-buffer no. 1: 0.3 M TRIS, 20% (v/v) methanol, pH 10.4 and placed on the anodic graphite plate. The next 3 layers of filterpaper were soaked in anodic-buffer no. 2: 25 mM TRIS, 20% (v/v) methanol pH 10.4. The polyacrylamide gel was placed over the membrane, then covered with 3 sheets wetted with cathodic-buffer: 40

Table I. *Summary of purification of tyrosinase from cultured human melanoma cells*

The enzyme activity is expressed as the sum of 2-S- and 5-S-L-cysteinyl-L-dopa formed per minute

Step	Protein (mg)	Tyrosinase activity (µmol)	Specific activity (µmol/mg)	Recovery (%)
1. Membranes solubilized by Triton X-100	72	39	0.54	–
2. Q-Sepharose chromatography	33	44	1.3	113
3. Phenyl-Sepharose chromatography	11	26	2.4	67
4. Tryptic digestion				
5. Con-A–Sepharose chromatography	2.2	19	8.6	49
6. Phenyl-Superose chromatography	0.21	14	67	36

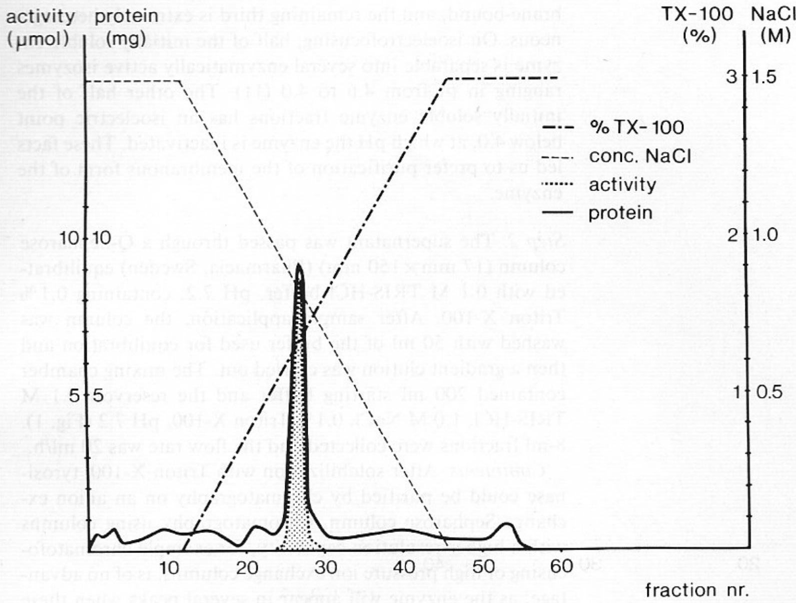

Fig. 2. Hydrophobic interaction phenyl-Sepharose chromatography of pooled tyrosinase containing fractions from Q-Sepharose chromatography. Activity and protein given per fraction.

mM 6-amino-*n*-hexanoic acid, 20% (v/v) methanol pH 9.4, finally, the cathodic graphite plate was placed on top.

Running conditions: 0.8 mA/cm² membrane for 70 min. Staining: The membrane was soaked i H_2O of Millipore quality for 5 min, and then stained in 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol 5 min, and destained in 50% (v/v) methanol, 10% (v/v) acetic acid 5 min. After destaining, the gel was rinsed in H_2O of Millipore quality, dried and kept frozen. The Coomassie Brilliant Blue band was cut out and sequenced on an Applied Biosystem model 470 sequenator (10).

Purification

A summary of a typical purification procedure is shown in Table I.

Step 1: About 2.5 g of cells in 30 ml 0.9% NaCl were homogenized with a Polytron PT 10/35 homogenizer (Kinematica, Kriens Luzern, Switzerland) 3×1 sec (level 4). The sample was centrifugated at 100000 *g* for one hour. To the pellet, 30 ml 0.1 M TRIS-HCl buffer, pH 7.2, was added and the sample rendered homogeneous by means of a glass homogenizer. Triton X-100 (scintillation grade, Merck) was added to

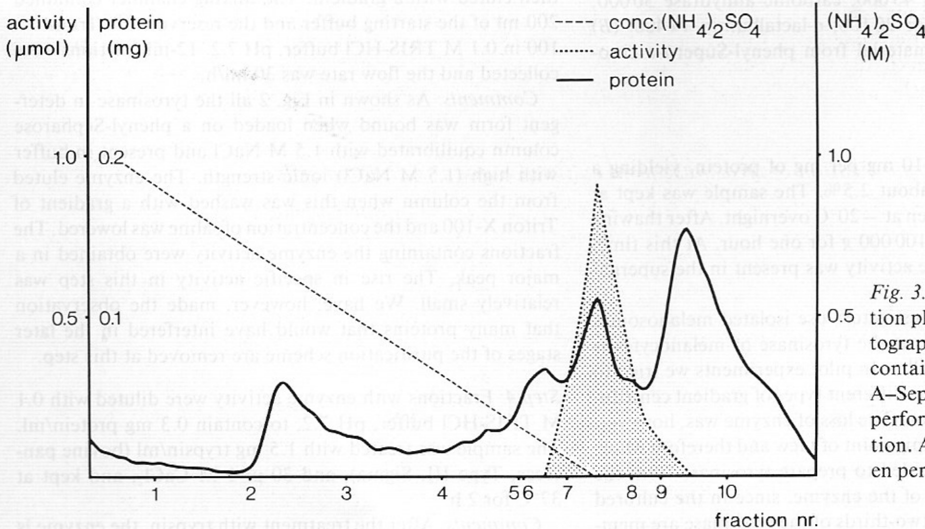

Fig. 3. Hydrophobic interaction phenyl-Superose chromatography of pooled tyrosinase-containing eluates from Con-A–Sepharose chromatography performed after tryptic digestion. Activity and protein given per ml.

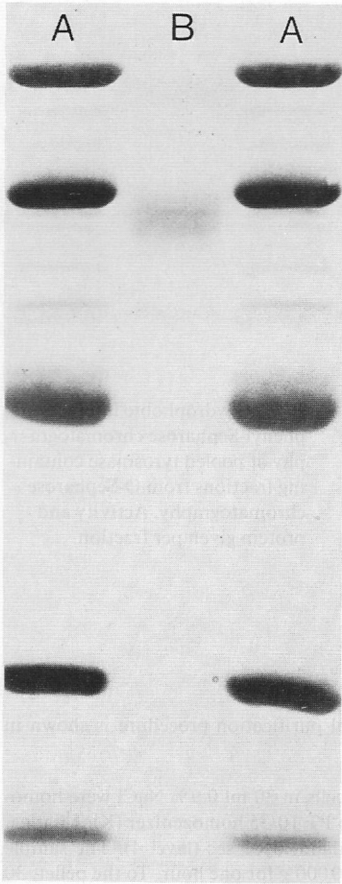

Fig. 4. Analysis of the tyrosinase-containing fractions from phenyl-Superose chromatography by SDS electrophoresis and subsequent blotting onto PVDF membrane. Staining for proteins with Coomassie Brilliant Blue R-250. (*A*) Calibration Kit proteins, phosphorylase b 94 000, bovine serum albumin 67 000, ovalbumin 43 000, carbonic anhydrase 30 000, soybean trypsin inhibitor 20 100, α-lactalbumin 14 400. (*B*) Tyrosinase-containing material from phenyl-Superose chromatography.

the suspension to give 10 mg per mg of protein, yielding a final concentration of about 2.5%. The sample was kept at 0°C for one hour and then at −20°C overnight. After thawing it was centrifugated at 100 000 *g* for one hour. At this time, practically all tyrosinase activity was present in the supernatant.

Comments: Many investigators use isolated melanosomes as the starting material, as the tyrosinase of melanocytes is enriched in these organelles. In pilot experiments we tried to isolate these organelles by different types of gradient centrifugation and phase partition. The loss of enzyme was, however, too large from an economic point of view and therefore whole cells were used. We decided to prepare tyrosinase from the membrane-bound form of the enzyme, since in the cultured human melanoma cells two-thirds of the tyrosinase are membrane-bound, and the remaining third is extremely heterogeneous. On isoelectrofocusing, half of the initially soluble enzyme is separable into several enzymatically active isozymes ranging in pI from 4.6 to 4.0 (11). The other half of the initially soluble enzyme fractions has an isoelectric point below 4.0, at which pH the enzyme is inactivated. These facts led us to prefer purification of the membranous form of the enzyme.

Step 2: The supernatant was passed through a Q-Sepharose column (17 mm×150 mm) (Pharmacia, Sweden) equilibrated with 0.1 M TRIS-HCl buffer, pH 7.2, containing 0.1% Triton X-100. After sample application, the column was washed with 50 ml of the buffer used for equilibration and then a gradient elution was carried out. The mixing chamber contained 200 ml starting buffer and the reservoir 0.1 M TRIS-HCl, 1.0 M NaCl, 0.1% Triton X-100, pH 7.2 (Fig. 1). 8-ml fractions were collected and the flow rate was 20 ml/h.

Comments: After solubilization with Triton X-100, tyrosinase could be purified by chromatography on an anion exchange Sepharose column. Chromatography using columns with a higher resolution capability, for example chromatofocusing or high pressure ion exchange columns, is of no advantage, as the enzyme will appear in several peaks when these methods are used. The Q-Sepharose column is an efficient step in the purification because not only irrelevant proteins but also other impurities such as lipids and melanin pigments were removed. These impurities would have prevented the efficient binding of the enzyme to subsequent columns. The recovery of 113% in this step probably reflects the imperfection of our tyrosinase method for enzyme determination in the very crude sample of step one.

Step 3: The fractions containing tyrosinase in step two were pooled and solid NaCl was added to give a final concentration of 1.5 M. The solution was then applied to a hydrophobic interaction column (10 mm×170 mm) containing phenyl-Sepharose (Pharmacia, Sweden), previously equilibrated with the 0.1 M TRIS-HCl buffer, pH 7.2, containing 1.5 M NaCl. After sample application the column was washed with 50 ml of the same buffer as that used for equilibration and then eluted with a gradient. The mixing chamber contained 200 ml of the starting buffer and the reservoir 3% Triton X-100 in 0.1 M TRIS-HCl buffer, pH 7.2. 12-ml fractions were collected and the flow rate was 30 ml/h.

Comments: As shown in Fig. 2 all the tyrosinase in detergent form was bound when loaded on a phenyl-Sepharose column equilibrated with 1.5 M NaCl and present in buffer with high (1.5 M NaCl) ionic strength. The enzyme eluted from the column when this was washed with a gradient of Triton X-100 and the concentration of saline was lowered. The fractions containing the enzyme activity were obtained in a major peak. The rise in specific activity in this step was relatively small. We have, however, made the observation that many proteins that would have interfered in the later stages of the purification scheme are removed at this step.

Step 4: Fractions with enzyme activity were diluted with 0.1 M TRIS-HCl buffer, pH 7.2, to contain 0.3 mg protein/ml. The sample was treated with 1.5 mg trypsin/ml (bovine pancreas, Type III, Sigma), and 30 µl 1 M $CaCl_2$, and kept at 37°C for 2 h.

Comments: After the treatment with trypsin, the enzyme is

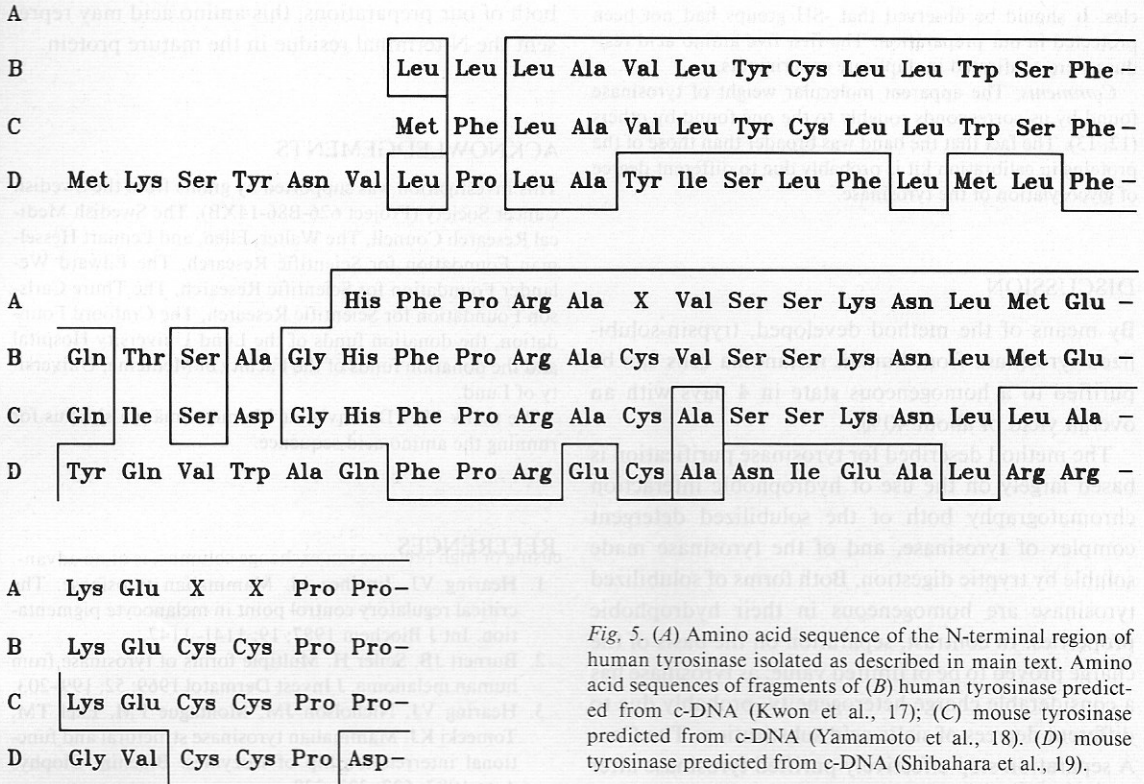

Fig. 5. (*A*) Amino acid sequence of the N-terminal region of human tyrosinase isolated as described in main text. Amino acid sequences of fragments of (*B*) human tyrosinase predicted from c-DNA (Kwon et al., 17); (*C*) mouse tyrosinase predicted from c-DNA (Yamamoto et al., 18). (*D*) mouse tyrosinase predicted from c-DNA (Shibahara et al., 19).

stable in solution in the absence of detergent. Several concentrations of trypsin and lengths of incubation were examined to ascertain the optimal conditions for digestion. If the digestion was interrupted too early, contaminants which could not be separated from tyrosinase in the subsequent steps remained. When more extensive digestion was carried out, loss of the enzyme occurred.

Step 5: The trypsinized sample was cleared by centrifugation at 30 000 *g* for 30 min, and then passed through a Concanavalin A–Sepharose column (19 mm × 35 mm) (Pharmacia, Sweden) equilibrated with 4 mM KH_2PO_4, 1 M KCl, pH 7.0. The column was washed with 5 ml of 4 mM KH_2PO_4, 1 M KCl, pH 7.0, and 5 ml of 4 mM KH_2PO_4, pH 7.0, and then eluted with 0.5 M methyl *α*-D-mannopyranoside, 4 mM KH_2PO_4, pH 7.0, in 4-ml fractions.

Comments: Tyrosinase binds tightly to Con-A–Sepharose, as it is a high mannose glycoprotein (12). Thus it cannot be removed with low concentrations of methyl *α*-D-mannopyranoside. Tyrosinase is, however, eluted in good yield by a 0.4 M methyl *α*-D-mannopyranoside solution.

Step 6: To the eluate with enzyme activity, ammonium sulphate was added to give a final concentration of 1.0 M. The enzyme was then passed through a FPLC hydrophobic interaction column HR 5/5 (Pharmacia, Sweden). The column had been equilibrated with 20 mM KH_2PO_4, 1 M $(NH_4)_2SO_4$, pH 7.2. The sample was applied with a superloop (50 ml) (Pharmacia, Sweden). After sample application, the column was washed with 5 ml of starting buffer and then eluted with a gradient:

buffer A: 1 M $(NH_4)_2SO_4$, 20 mM KH_2PO_4 pH 7.2
buffer B: 20 mM KH_2PO_4 pH 7.2, 0–100 % in 40 min, flow rate 0.5 ml/min.

Comments: The enzyme applied was in most cases found in a distinct, symmetrical peak (Fig. 3). The specific activity had risen considerably. Small contaminants of other proteins were present in the tyrosinase peak, however. In a set of experiments, we tried to eliminate the contaminants by means of gel filtration, but even when chromatography on FPLC–Superose was performed, traces of other proteins remained.

Step 7: To get a completely purified enzyme, the materials corresponding to the peak containing tyrosinase activity in step 6 were electrophoresed. The eluates were concentrated using microconcentrators (Centricon 30 Amicon, Mass., USA), to get a volume suitable for application. After electrophoresis the proteins were blotted onto a PVDF membrane and stained with Coomassie Brilliant Blue R-250. One rather broad protein band, M_r 66 000, could be detected on the membrane loaded with the protein containing the tyrosinase activity (Fig. 4). This band was cut out and sequenced by automated Edman degradation. The result from a partial sequence determination (20 cycles) of the aminoterminal portion by automated Edman degradation is seen in Fig. 5. No relevant amino acids could be identified in three of the cy-

cles. It should be observed that -SH groups had not been protected in our preparation. The first five amino acid residues were confirmed in duplicate experiments.

Comments: The apparent molecular weight of tyrosinase found by us corresponds roughly to the one found by others (12, 13). The fact that the band was broader than those of the proteins in calibration kit is probably due to different degree of glycosylation of the tyrosinase.

DISCUSSION

By means of the method developed, trypsin-solubilized tyrosinase from human melanoma cells can be purified to a homogeneous state in 4 days with an overall yield of about 40%.

The method described for tyrosinase purification is based largely on the use of hydrophobic interaction chromatography both of the solubilized detergent complex of tyrosinase, and of the tyrosinase made soluble by tryptic digestion. Both forms of solubilized tyrosinase are homogeneous in their hydrophobic properties. In contrast, separation on the basis of the charge proved to be of limited value, as tyrosinase has a considerable charge heterogeneity, probably due to different degrees of sialic acid substitution. The Con-A separation step effectively purified tyrosinase after tryptic digestion. The fact that tyrosinase is stable to proteinases and can be brought into a soluble form with, for example, trypsin, has been used by several groups in their preparation of tyrosinase from mammalian sources (5, 14, 15, 16). The stability to trypsin is probably due to several disulfide bonds in the N-terminal portion of the protein.

The amino acids of the N-terminus that were identified in our preparation are identical with the residues 7–26 in a sequence predicted by Kwon et al. (17) from a human tyrosinase c-DNA clone. Since -SH groups were not protected in our preparation, cysteine should not be recovered in our assay. The positions where we found no amino acid residue correspond to cysteine in the sequence presented by Kwon et al. Furthermore the residues in our sequence are almost identical with residues 19–38 of a protein predicted from a mouse c-DNA clone by Yamamoto et al. (18). Our amino acid residues 2–5 were also found by Yamamoto et al. on sequencing a cyanogen bromide fragment obtained from purified mouse tyrosinase. The sequence suggested by Shibahara et al. for mouse tyrosinase (19) also shows similarity to that found by us. As seen in Fig. 5, different N-terminal residues are suggested by all c-DNA sequences presented. As histidine was found to be the N-terminal in both of our preparations, this amino acid may represent the N-terminal residue in the mature protein.

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